AMENDMENTS

IN THE SPECIFICATION:

8-13-09

On page 1, line 2:

B1 This application ~~is application~~ is a divisional of U.S. Serial No. 08/474,892, filed June 7, 1995, which is a divisional of U.S. Serial No. 07/973,588, filed November 9, 1992, which is a continuation of U.S. Ser. No. 07/438,544, filed November 20, 1989, now abandoned, which was a continuation-in-part of U.S. Ser. No. 07/273,373, filed November 18, 1988, now abandoned.

On page 4, line 7:

B2 [Figure 1] Figures 1A through 1G shows the nucleotide sequence for the RGB-2 cDNA and deduced amino acid sequence of the rat D_2 dopamine receptor. The nucleotide sequence is numbered beginning with the first methionine of the long open reading frame. Nucleotide numbering appears beneath the nucleotide sequence at the right-hand end of each line. The deduced amino acid sequence is shown above the nucleotide sequence. The double underline denotes the small open reading frame in the 5' untranslated region. Postulated N-linked glycosylation sites are indicated by asterisks. Putative protein kinase A phosphorylation sites have a line above them. The intron splice junction is designated by an arrow. The poly A adenylation site is underlined.

On page 5, line 6:

B3 [Figure 4] Figures 4A through 4C illustrates the binding of ^{3}H-spiperone to membranes from L-RGB2Zem-1 cells.

On page 5, line 8:

B4 [a) 1: Saturation] Figure 4A-1 shows isotherms of the specific binding of ^{3}H-spiperone to membranes prepared from L-RGB2Zem-1 cells and rat striatum. Results are shown from one of four independent experiments.

On page 5, line 12:

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B5 [2: Scatchard] Figure 4A-2 shows Scatchard transformation of the data .

On page 5, line 13:

[b) Competitions] Figure 4B shows competition curves using L-RGB2Zem-1 membranes. Representative curves are shown for inhibition of specifically bound ^{3}H-spiperone by drugs in membranes from L-RGB2Zem-1 cells. Each drug was tested 3 times.

On page 5, line 17:

B6 [c) Table] Figure 4C is a table of K_i values for L-RGB2Zem-1 and rat striatum. Results are geometric means of 3 experiments in which 0.5 nM ^{3}H-spiperone was inhibited by various concentrations of unlabeled drug. For some drugs, inhibition curves in rat striatal tissue were fit best by assuming the presence of two classes of binding sites. The proportions of binding sites with high or low affinity for inhibitor are shown in parentheses. K_i values for the class of binding sites representing 10-25% of specific binding were calculated by assuming that the radioligand was binding to serotonin receptors with a K_d value of 1 nM.

On page 5, line 29:

[Figure 5 shows (a) a] Figure 5A shows [(a)] a hydrophobicity plot of the amino acid sequence shown in [Figure 1] with Figures 1A through 1G; and Figure 5B shows [(b)] a hydrophobicity plot of the amino acid sequence of the β_2-adrenergic receptor. The transmembrane regions are marked by the Roman numerals.

On page 5, line 34:

[Figure 6 shows] Figures 6A through 6E show a calculated restriction map of a 2477 base EcoRI fragment of the nucleic acid sequence shown in [Figure 1] Figures 1A through 1G.

On page 6, line 1:

B7 [Figure 7 shows] Figures 7A through 7C show a partial sequence of a human D_2 dopamine receptor, the middle amino acid sequence shown being the correct one.

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On page 6, line 22:

[Figure 9 shows the] Figures 9A and 9B show inhibition of radioligand binding by agonists. Results are plotted as specific binding, expressed as a percentage of specific binding in the absence of competing drug, versus the log of the concentration of competing drug. Membranes were prepared from LZR1 cells as described in the text.

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On page 6, line 28:

[A. Curves] Figure 9A shows curves from a single experiment are shown for inhibition of the binding of ^{3}H-spiroperidol by agonists. Each drug was tested twice. In this experiment, the free concentration of ^{3}H-spiroperidol was 230 pM, and the K_D value for ^{3}H-spiroperidol was 60 pM. K_I values and Hill coefficients in this experiment were 5 nM and 1.05 for bromocriptine, respectively, 790 nM and 0.89 for (-)3-PPP, 8 μM and 1.0 for quinpirole, 31 μM and 1.05 for (+)3-PPP, and 0.3 mM and 0.72 for LY181990. .

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On page 7, line 1:

[B. Results are shown] Figure 9B shows from one of four independent experiments in which the effect of GTP and NaCl on the inhibition of ^{3}H-spiroperidol binding by DA was determined. Concentrations of ^{3}H-spiroperidol ranged from 323 to 498 pM. In this experiment, the concentration of radioligand was 323 pM. Open circles represent inhibition by DA in the presence of 0.1 mM GTP and 120 mM NaCl, whereas closed circles represent inhibition in the absence of added GTP and NaCl. IC_{50} values and Hill coefficients in this experiment were 29 μM and 0.65, respectively, in the absence and 115 μM and 1.03 in the presence of GTP and NaCl. .

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On page 7, line 13:

[Figure 10 shows the] Figures 10A and 10B show inhibition of adenylate cyclase activity in LZR1 cells. Agonists were tested for inhibition of adenylate cyclase activity in membranes prepared from LZR1 cells. Approximately 50 to 100 μg of protein was used in each assay. Results are shown as ^{32}P-cAMP/mg of protein/min., expressed as a percentage of total activity in the presence of 10 μM forskolin. Representative dose-response curves are shown for six drugs, each tested at least three

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times. Data are plotted as enzyme activity versus the log of the concentration of drug. No curve is plotted for the data for (-)3-PPP, since no inhibition was observed. In the experiments shown in [this figure] these Figures, basal and forskolin-stimulated activity ranged from approximately 0.8 to 1.5 and 8.5 to 15.8 pmol/mg of protein/min., respectively . B11

On page 8, line 5:

[Figure 12 shows the] Figures 12A through 12C show reversal of dopamine inhibition by pertussis toxin pretreatment. Data presented for membrane adenylate cyclase activity represent means (x) with standard error (S.E.) and % inhibition (% Inh.) below. % Inhibition was calculated from the equation 100 x $\{1-(S-B/I-B_I)\}$ where B, S, and I are values of basal activity, activity in the presence of stimulator (S) or inhibitor (I), respectively and B, is basal activity in the presence of inhibitor. Results were obtained from parallel assays in controls and cells pretreated (16 h, 50 ng/ml) with pertussis toxin (indicated as +P.T.). B12

On page 8, line 16:

[(A) Adenylate Cyclase] Figure 12A shows the results of adenylate cyclase assays. Membranes for cyclase assay were exposed acutely to 10 μM forskolin (FSK) or 100 μM dopamine (DA), and adenylate cyclase activity expressed as pmol/mg protein/min. B13

On page 8, line 20:

[(B) Intracellular cAMP.] Figure 12B shows the results of intracellular cAMP assays. Cells were treated acutely with VIP (200 nM) and dopamine (1 μM) and cAMP accumulation (expressed as pmol/dish) was measured in cell extracts. B14

On page 8, line 24:

[(C) Extracellular cAMP.] Figure 12C shows the results of extracellular cAMP assays. Media samples from the same dishes of cells were assayed for cAMP accumulation expressed as pmol/dish. B15

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On page 8, line 27:

B16 [Figure 13 shows the] Figures 13A through 13C show expression of specific dopamine-D_2 receptor mRNA and specific binding in GH_4ZR_7 transfectant cells.

On page 8, line 30:

B17 [(A) Northern] Figure 13A shows the results of Northern blot analysis of GH_4C_1 cell total RNA (20 µg/lane). Y-axis indicate the migration of RNA molecular weight standards (kb).

On page 8, line 33:

B18 [(B) (1) Specific] Figure 13B-1 shows the results of specific binding of ^{3}H-spiperone to membranes prepared from GH_4ZR_7 cells was characterized by saturation analysis (see "Experimental Procedures", Example 2). Data from one of four independent experiments are plotted as specifically bound radioligand (ordinate) versus corrected free radioligand concentration (total added minus total bound). Calculated K_D and B_{max} values for this experiment were 60 pM and 1165 fmol/mg protein.

On page 9, line 5:

B19 [(B) (2) Transformation] Figure 13B-2 shows the results of transformation of the data by the method of Scatchard which are plotted as specific bound/free (Y-axis) vs. specific bound concentrations of .sup.3H-spiperone (X-axis).

On page 9, line 9:

B20 [(C) Displacement] Figure 13C shows the results of displacement of specific ^{3}H-spiperone binding by dopamine: effect of GTP/NaCl. GH_4ZR_7 cell membranes were incubated with ^{3}H-spiperone (0.47 nM) and indicated concentrations of dopamine (X-axis) in the absence (○) or presence (•) of 100 µM GTP/120 mM NaCl. Results are shown for one of four experiments. Calculated IC_{50} and Hill coefficient values for dopamine in the experiment shown were 16 µM and 0.61 in the absence of GTP/NaCl, and 56 µM and 0.85 in the presence of GTP/NaCl.

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[Figure 14 shows the] Figures 14A through 14C show inhibition of cAMP accumulation and PRL release by dopamine in GH_4ZR_7 cells. Incubations were performed in triplicate as described in "Experimental Procedures", Example 2.

On page 9, line 23:

["(A) Inhibition] Figure 14A shows the results of assays showing inhibition of extracellular cAMP accumulation by dopamine. Parallel dishes of GH_4C_1 and of extracellular cAMP accumulation by dopamine. Parallel dishes of GH_4C_1 and GH_4ZR_7 cells were incubated with concentrations of VIP, dopamine (D), and (-)-sulpiride (-S) of 250 nM, 10 μM and 5 μM, respectively. Untreated controls are denoted as "C". Media were collected and assayed for cAMP (ordinate) expressed as pmol/dish. cells were incubated with concentrations of VIP, dopamine (D), and (-)-sulpiride (-S) of 250 nM, 10 μM and 5 μM, respectively. Untreated controls are denoted as "C". Media were collected and assayed for cAMP (ordinate) expressed as pmol/dish.

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On page 9, line 30:

[(B) Inhibition] Figure 14B shows the results of assays showing inhibition of intracellular cAMP accumulation by dopamine in GH_4ZR_7 cells. Cell extracts were assayed for cAMP, expressed on the ordinate. Drug concentrations were as in (A), except (+)-sulpiride (+S), 5 μM.

On page 9, line 35:

[(C) Inhibition] Figure 14C shows the results of assays showing inhibition of stimulated PRL release by dopamine in GH_4ZR_7 cells. Media samples were assayed for PRL (ordinate) after the indicated treatments. The concentrations of VIP, TRH, dopamine (D), and (-)-sulpiride (-S) were 200 rdM, 200 nM, 100 nM, and 2 μM, respectively.

On page 10, line 4:

[Figure 15 shows] Figures 15A and 15B show dose-response relations for dopamine inhibition of basal and VIP-enhanced cAMP accumulation in GH_4ZR_7.

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On page 10, line 7:

[(A) Basal] Figure 15A shows basal intra- (•) and extracellular (○) cAMP accumulation in the presence of indicated concentrations of dopamine. Basal cAMP levels in the absence of dopamine were 22 ±6 pmol/dish (intracellular) and 12.4 ±.0.6 pmol/dish (extracellular). EC_{50} values for dopamine actions were 4.9 nM (intracellular) and 8.5 nM (extracellular).

On page 10, line 14:

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[(B) VIP-enhanced] Figure 15B shows VIP-enhanced intra- (•) and extracellular (.) cAMP accumulation in the presence of indicated dopamine concentrations. VIP (250 nM)-enhanced levels of intra- and extracellular cAMP (in the absence of dopamine) were 145±1.2 pmol/dish and 146±2.8 pmol/dish, and basal cAMP levels were 35±1.6 pmol/dish and 15±0.2 pmol/dish, respectively. EC_{50} values for dopamine -inhibition were 5.5 nM (intracellular) and 5.8 nM (extracellular).

On page 11, line 6:

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[Figure 17 shows the] Figures 17A and 17B show inhibition of adenylate cyclase by dopamine-D_2 agonists. Inhibition of adenylate cyclase activity was assessed in the presence of 10 μM forskolin. Data are plotted as the mean of triplicate assays, with enzyme activity expressed as a percentage of total activity versus the logarithm of drug concentration. Average basal adenylate cyclase activity was 4.6±0.2 pmol/mg protein/min and total forskolin-stimulated activity was 63.8±0.2 pmol/mg protein/min. EC_{50} values and maximal inhibition for the experiments shown were 79 nM and 57%, respectively, for dopamine, 200 nM and 49% for quinpirole, 5 nM and 23% for bromocryptine, and 600 μM and 40% for (+)-3-PPP.

On page 11, line 19:

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[Figure 18 shows] Figures 18A through 18J show the nucleotide sequence of the human pituitary dopamine-D_2 receptor cDNA. The deduced amino acid sequence is indicated above the human cDNA. Below is the nucleotide sequence of the cloned rat cDNA (see Reference 9, Example 3) and the amino acids which differ between the two clones. Boxed regions, numbered I-VII, represent the putative transmembrane domains. Triangles indicate the exon/intron splice junctions;

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B25 a period is one missing base pair; asterisks identify potential –linked glycosylation sites and targets of protein kinase A phosphorylation are underlined. The polyadenylation signal is double underlined.

On page 14, line 27:

B26 [Figure 1 shows] Figures 1A through 1G show the nucleotide sequence of 2455 bases for the RGB-2 cDNA. The longest open reading frame in this cDNA codes for a 415 amino acid protein (relative molecular weight (M_r=47,064)) also shown in the figure. This molecular weight is similar to that reported for the deglycosylated form of the D_2-dopamine receptor as determined by SDS polyacrylamide gel electrophoresis (11). An in-frame dipeptide which is 36 bases upstream from the putative initiation site is found in the 5' untranslated region of the RGB-2 cDNA. A small open reading frame has been observed in the 5' untranslated sequence of the β_2-AR mRNA (9).

On page 15, line 6:

B27 Several structural features of the protein deduced from the RGB-2 cDNA demonstrate that it belongs to the family of G protein-coupled receptors. The hydrophobicity plot of the protein sequence [Figure 5] Figure 5A shows the existence of seven stretches of hydrophobic amino acids which could represent seven transmembrane domains (8). Moreover, the primary amino acid sequence of RGB-2 shows a high degree of similarity with other G protein-coupled receptors (FIG. 2). The regions of greatest amino acid identity are clustered within the putative transmembrane domains. Within these domains the RGB-2 protein has a sequence identity of 50% with the human α_2-adrenergic receptor (12), 42% with the human G-21 receptor (13), 38% with the hamster β_2-AR -adrenergic receptor (9), 27% with the porcine M.sub.1 receptor (14), and 25% with the bovine substance K-receptor (15).

On page 18, line 23:

B28 Membranes prepared from control Ltk- cells showed no (+)-butaclamol- or sulpiride-displaceable binding of ^{3}H-spiperone. Binding of ^{3}H-spiperone to membranes prepared from L-RGB2Zem-1 cells was saturable with a K_d value of 48 pM [Figure 4a] Figure 4A. This value

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agrees with that observed for binding of ^{3}H-spiperone to rat striatal membranes in parallel experiments (52 pM). In the experiment shown in [Figure 4a] Figure 4A, K_d and B_{max} values for membranes prepared from L-RGB2Zem-1 were 40 pm and 876 fmol/mg of protein, whereas the corresponding values in striatal membranes were 37 pm and 547 fmol/mg of protein. The density of binding sites as determined in four experiments was 945 fmol/mg of protein in L-RGB2Zem-1 membranes and 454 fmol/mg of protein in rat striatal membranes.

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On page 18, line 36:

The binding of ^{3}H-spiperone to membranes from L-RGB2Zem-1 cells was inhibited by a number of drugs and the resulting K_i values closely matched those obtained using striatal membranes [Figures 4b, 4c] Figures 4B and 4C. The D_2 antagonists (+)-butaclamol and haloperidol were the most potent inhibitors, followed by sulpiride. The D_1 dopamine antagonist SCH 23390 and the serotonin $5HT_2$ antagonist ketanserin were much less potent at blocking ^{3}H-spiperone binding. The binding appeared to be stereoselective as (+)-butaclamol was much more potent than (-)-butaclamol at inhibiting binding. In these experiments the absolute affinities of dopaminergic antagonists and the rank order of potency of the drugs (spiperone>(+)-butaclamol>haloperidol>sulpiride>>(-)-butaclamol) agree closely with previously published values for the D_2 dopamine receptor (2).

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On page 19, line 19:

All binding data for L-RGB2Zem-1 membranes were fit best by assuming the presence of only one class of binding sites. On the other hand, inhibition by several drugs of ^{3}H-spiperone binding to rat striatal membranes was fit best by assuming the presence of two classes of binding sites. Thus, SCH 23390 and ketanserin inhibited 10-20% of ^{3}H-spiperone binding to rat striatal membranes with high affinity ([Figure 4c] Figure 4C). In rat striatal membranes, inhibition of radioligand binding by sulpiride was fit best by one class of binding sites, but 10-15% of the (+)-butaclamol-displaceable binding was not inhibited by sulpiride at the concentrations used. It seems likely that the binding sites with high affinity for ketanserin and SCH 23390 and which are not displaced by sulpiride represent binding of ^{3}H-spiperone to $5HT_2$ serotonin receptors in rat

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striatal membranes. Binding of SCH 23390 to $5HT_2$ receptors has been described previously (22). In rat striatal membranes, the apparent affinity of drugs for D_2 dopamine receptors, the class of binding sites comprising 80-90% of ^{3}H-spiperone binding, was indistinguishable from the apparent affinity of drugs to membranes prepared from L-RGB2Zem-1 cells ([Figure 4c] Figure 4C).

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On page 20, line 24:

The nucleic acid sequence shown in [Figure 1] Figures 1A through 1G can be inserted into a wide variety of conventional and preferably commercially available plasmids, e.g., using EcoRI sites or other appropriate sites. See, e.g., FIG. 6 for a restriction map of the sequence of [Figure 1] Figures 1A through 1G .

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On page 20, line 32:

Dopamine receptor genes of this invention, particularly mammalian D_2 dopamine receptor genes, based on this disclosure, can now be routinely made, isolated and/or cloned, using many conventional techniques. For example, the procedure disclosed herein can be substantially reproduced for libraries containing dopamine receptor DNA sequences. Alternatively, oligonucleotide probes can be routinely designed, e.g., from the sequences of [Figure 1] Figures 1A through 1G and/or the omitted introns, which are selective for dopamine receptor genes, especially for mammalian dopamine D_2 receptor genes. These can be used to screen nucleic acid libraries containing dopamine receptor nucleic acid sequences. Sequences in these libraries hybridizing to the probes, especially to all of a plurality of such probes (e.g. 2 or 3), will be DNA sequences of this invention with high probability. Of course, it is also possible to synthesize the sequence of [Figure 1] Figures 1A through 1G or any fragment thereof using conventional methods.

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On page 22, line 20:

These polypeptides can be expressed from the nucleic acids of this invention by procaryotic or eucaryotic hosts, e.g., bacterial, yeast or mammalian cells in culture, using fully conventional transformation or transfection (e.g., via calcium phosphate for mammalian cells) techniques. The products of such expression in vertebrate (e.g., mammalian and avian) cells are especially

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advantageous in that they are produced free from association with other human proteins or contaminants with which they may be associated in natural form. Preferred hosts for expression are mammalian and include for example mouse Ltk$^-$ cells, hamster CHO cells, mouse GH_4 cells, mouse C_6 cells, mouse/rat NG108-15 cells and mouse AtT20 cells. For example, when the gene of [Figure 1] Figures 1A through 1G is transfected into the commercially available growth hormone GH_4 cells, modulation of the cAMP second messenger system has been observed. Preferred vectors include pZem or pRSV or viral vectors such as vaccinia virus and retroviruses.

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On page 22, line 34:

Also included in this invention are polypeptides, synthetic or otherwise, duplicating the amino acid sequence of [Figure 1] Figures 1A through 1G and/or of the dopamine receptors per se of this invention, or only partially duplicating the same. These wholly or partially duplicative polypeptides will preferably also retain the biological and/or immunological activity of the dopamine receptor per se. Also included within the scope of this invention are the monoclonal and polyclonal antibodies (generatable by conventional techniques and preferably labelled) which are immunoreactive with such polypeptides.

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On page 23, line 25:

Preferred partial polypeptides (fragments) are those including at least a portion of the sequences located in the hydrophobic transmembrane domains V, VI and VII, shown in FIG. 2. These are the likely locations of the ligand binding site(s), particularly domain VII. The third cytoplasmic loop is also an important fragment area; e.g., G-protein binding requires this location as well as domains V and VI. Where it is desired to have an antibody highly specific to a particular dopamine receptor, a fragment generating such an antibody will be selected from the highly unique region between transmembrane regions V and VI, i.e., the third cytoplasmic loop, or the C-terminal domain, both of which have low homology with other receptors, and/or the antibodies will be selected to be specific to an epitope in these regions. Another receptor/gene specific region is that of the intron sequences, e.g., those for RGB-2, mentioned above. Particularly preferred peptides which have been synthesized are (referring to the amino acid numbers of [Figure 1] Figures 1A

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through 1G): (A) 2-13; (B) 182-192; (C) 264-277; (D) 287-298; and (E) 404-414. These are selected based on the following principles: the known antigenicity of peptides containing a large number of Pro residues (B/C/D); coverage of the N and C termini (A) and (E); the ability to direct antibodies towards an extracellular domain (receptor reaction region) which will be effective to block the receptor reaction (A/C/D). Antibodies to these fragments are raised conventionally, e.g., monoclonals by fully conventional hybridoma techniques.

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On page 25, line 20:

Modified proteins which do not retain the mentioned biological activity and/or the corresponding DNA sequences will also be useful, e.g., in various assays of this invention. In a particularly preferred such modification, the transmembrane domain V, VI, or VII or the third cytoplasmic loop will be deleted or rendered inactive, e.g., by sequence modification. Deletion of the glycosylation sites shown in [Figure 1] Figures 1A through 1G is also a useful variant for expression of the polypeptide, e.g., in yeast cells.

B36

On page 25, line 34:

As mentioned above, it is well established that significant portions of the DNA sequence encoding a dopamine receptor are conserved in various mammalian species. Consequently, using only routine experimentation, a skilled worker can readily screen a DNA genomic library or, preferably, a cDNA library, e.g., from the brain of a given mammal, for the presence of other dopamine receptor genes, especially D_2 dopamine receptor genes, using probes manufactured in accordance with the details of the sequences shown herein, including the 5' flanking, the intronic and the structural gene sequences shown in [Figure 1] Figures 1A through 1G and the human sequence of [Figure 7] Figures 7A through 7C. Probes will preferably be selected from the seven highly conserved transmembrane domains shown in FIG. 2, preferably domains VI and VII. Such a routine screening will identify clones which hybridize with the probes. From these, dopamine receptors can routinely be selected, e.g., using the techniques described herein. With respect to human D_2 dopamine receptors, particularly useful sources include, for cDNA, striatum, pituitary, neuroblastoma, kidney, placenta cells, etc., and, for genomic DNA, liver, placenta cells, etc. For

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B37 primates, e.g., rhesus monkeys, particularly useful genomic DNA or cDNA libraries include brain, kidney and placenta cells.

On page 26, line 11:

With respect to human D_2 dopamine receptor genes, the partial sequence-shown [Figure 7] Figures 7A through 7C has been identified by conventionally screening, under the stringent hybridization conditions described above for the probing by the 0.8 kb EcoRI-PstI fragment of rat brain cDNA in λgt10, a pituitary cDNA library using a probe which is the full length rat cDNA, RGB-2. The cDNA libraries mentioned herein were prepared by fully conventional methods, e.g., B38 as described in the references cited above, e.g., Davis et al. This sequence or fragments thereof can also be useful as a probe, for example, to screen conventional libraries as mentioned above for human dopamine receptor genes in accordance with the foregoing and other fully conventional procedures. As can be seen by comparing the sequence of [Figure 7] Figures 7A through 7C with the sequences shown in [Figure 1] Figures 1A through 1G and Figure 2 above, the partial human sequence of [Figure 7] Figures 7A through 7C has high homology with RGB-2 beginning at amino acid no. 259 of [Figure 1] Figures 1A through 1G .

NE On page 30, line 4:

The polypeptides of this invention can also be used in, e.g., competitive binding assays, to test for the affinity thereto of candidate chemical substances such as drugs, e.g., affinity (e.g., agonistic or antagonistic) to D_2 dopamine receptors. Such procedures can be carried out, e.g., as pharmaceutical screening tests, using fully conventional procedures, analogous to those described herein and/or to known protocols based on natural sources of dopamine receptors, e.g., analogous to known tests for inhibition of the binding of tritiated dopamine agonists and antagonists to striatal receptors per the methods of Schwarcz et al., J. Neurochemistry, 34 (1980), 772-778 and Creese et al., European J. Pharmacol., 46 (1977), 377-381, and to those for other receptors. It is also possible to screen substances for ability to modify or initiate a response which is triggered by ligand [bonding] binding to a dopamine receptor, e.g., cellular responses such as modulation of second messenger systems. Such analyses can utilize cells of this invention transformed with nucleic acid

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sequences of this invention.

NE On page 50, line 13:

The abbreviations used are: PRL, prolactin; GH, growth hormone; $[Ca^{++}]_i$, cytosolic free calcium concentration; VIP, vasoactive intestinal peptide; TRH, thyrotropin-releasing hormone; IBMX, 3-isobutyl-1-methyl xanthine; K_D, equilibrium dissociation constant; EC_{50} (IC_{50}), concentration required to elicit a half-maximal effect (inhibition).

NE On page 60, line 4:

Dopamine Actions on cAMP and PRL Levels: To test directly the function of the expressed dopamine-D_2 receptor clone, the actions of dopamine on cellular cAMP levels were measured. These assays were conducted in the presence of 100 μM IBMX, to inhibit phosphodiesterase activity in these cells (22). Thus, the observed changes in cAMP levels reflect changes in the rate of synthesis of cAMP rather than changes in its degradation. Dopamine actions on basal cAMP levels were measured, as well as dopamine inhibition of VIP-enhanced levels of cAMP. GH_4C_1 cells respond to VIP with an enhancement of cAMP accumulation (FIG. 14A) as described by others (22, 26). Dopamine had no effect on extracellular cAMP levels in wild-type GH_4C_1 cells, whether VIP was omitted or present during the incubation. This result is consistent with the lack of D_2 dopamine receptor mRNA and binding in GH_4C_1 cells [Figure 13] Figures 13A through 13C, and indicates that these cells also lack a detectable D_2 dopamine response since dopamine does not elevate cAMP concentrations. In media from GH_4ZR_7 cells, dopamine inhibited both basal cAMP levels (by 50-70%), and reduced VIP-enhanced cAMP to basal levels. These actions of dopamine to reduce cAMP levels were consistently observed in all experiments and dopamine was equally effective in lowering intracellular cAMP levels (FIG. 14B). As observed previously in GH_4C_1 cells (22), both intra- and extracellular cAMP levels change in parallel, although changes in extracellular cAMP may be more pronounced due to lower recovery of extracted intracellular cAMP. Dopamine actions on cAMP accumulation were blocked by (-)-sulpiride, a highly selective dopamine-D_2 antagonist, whereas the inactive stereoisomer, (+)-sulpiride, did not block dopaminergic inhibition of cAMP accumulation. Stereo-selective blockade by sulpiride suggested that inhibition of cAMP levels in

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GH_4ZR_7 by dopamine was mediated by activation of a dopamine-D_2 receptor not present in wild-type GH_4C_1 cells.

On page 61, line 7:

To examine whether concentrations required for biological response correlated with affinity for the dopamine-D_2 receptor, dose-response relations were examined for dopamine actions on cAMP levels [Figure 15] Figures 15A and 15B. Dopamine potently inhibited intra- and extracellular levels of cAMP with similar EC_{50} values. Furthermore, dopamine inhibited both basal and VIP-enhanced cAMP accumulation with EC_{50} values of 8±2 nM and 6±1 nM, respectively. These data demonstrate that dopamine inhibits both basal and stimulated cAMP accumulation with approximately equal potency. The high potency of these inhibitory actions of dopamine supports the assertion that GH_4ZR_7 cells express a functional dopamine-D_2 receptor.

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On page 62, line 8:

Inhibition of Adenylate Cyclase: To assess directly inhibition of adenylate cyclase activity by dopamine receptor agonists, the conversion of ^{32}P-ATP to ^{32}P-cAMP was measured in membranes prepared from GH_4ZR_7 cells ([Figure 17] Figures 17A and 17B). Dopamine inhibited total forskolin (10 μM)-stimulated activity by 45% with an average EC_{50} value of 0.36 μM (n-5). As observed in pituitary (11) and striatal (28) membranes, bromocryptine behaved as partial agonist, maximally inhibiting enzyme activity by 23% (EC_{50} = 6 nM). Inhibition of adenylate cyclase activity by selective D_2-agonists was stereo-selective. For example, quinpirole inhibited forskolin-stimulated cyclase activity by 41% (EC_{50} = 0.32 nM), whereas LY181990, the inactive (+)-enantiomer of quinpirole, cause no consistent reduction in enzyme activity. Similarly, (+)-3-PPP (EC_{50} = 0.86 nM) was as efficacious as dopamine, whereas the enantiomer (-)-3-PPP did not consistently reduce adenylate cyclase activity. VIP also stimulated adenylate cyclase activity in GH_4ZR_7 cell membranes, as reported for wild-type GH_4C_1 cell membranes (29). Total activity stimulated by 200 nM VIP was 22±7 pmol/mg protein/min (n=3), and VIP-enhanced activity was inhibited 41% by dopamine (100 μM), compared to 50-55% inhibition in rat anterior pituitary membranes (11). No effect of dopamine on basal adenylate cyclase activity was observed in these preparations.

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B40 Nevertheless, inhibition by dopamine of forskolin- or VIP-stimulated adenylate cyclase activity provides a likely mechanism for inhibition of cAMP accumulation by dopamine in GH_4ZR_7 cells.

NE On page 63, line 2:

Pertussis Toxin Sensitivity: Sensitivity to pertussis toxin is a hallmark of receptors, such as the dopamine-D_2 receptor (6-12, 15), which couple to inhibitor G proteins (e.g., G_i or G_0) to induce responses. Pretreatment of GH_4ZR_7 cells with pertussis toxin for 16 h ([Figure 12] Figures 12A through 12C) uncoupled dopamine-mediated inhibition of forskolin-stimulated membrane adenylate cyclase activity, and abolished inhibition of basal and VIP-stimulated cAMP accumulation by dopamine. The concentration of pertussis toxin and incubation time used produce maximal blockage of somatostatin responses in wild-type cells (30), and the dopamine responses were almost completely inhibited under these conditions. By contrast, basal and VIP-stimulated cAMP accumulation, as well as basal and forskolin-stimulated cyclase activity, were not significantly altered by pertussis toxin pretreatment. These data support the assertion that the expressed cDNA clone codes for a dopamine-D_2 binding site which is functionally coupled to inhibitory G proteins present in GH_4 cells, and thus represents a bona fide receptor.

On page 64, line 21:

The presence of dopamine-D_2 binding in the GH_4ZR_7 transfectant correlated with potent and powerful inhibition of cAMP accumulation and PRL release, as well as inhibition of forskolin stimulated adenylate cyclase activity, actions of dopamine not observed in GH_4C_1 cells. These inhibitory actions of dopamine match exactly the known physiological actions of dopamine in pituitary lactotrophs (1, 2). In particular, dopamine controls PRL secretion and cAMP accumulation in lactotrophs such that stimulation of these processes does not occur unless dopamine concentrations decrease to low levels (1,3-5). Similarly, in the present of maximal concentrations of dopamine, VIP does not enhance cAMP levels or PRL secretion in GH_4ZR_7 cells ([Figure 14] Figures 14A through 14C). The potency of dopamine inhibition of basal and VIP-enhanced cAMP accumulation in GH_4ZR_7 cells ([Figure 15] Figures 15A and 15B) was in the range of concentration

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expected for lactotrophs, given that dopamine concentrations in hypophyseal portal blood vary from 7 nM in female rates during proestrous, to 20 nM during estrous, and are 3 nM in male rats (31). Detailed analysis of the pharmacology of dopamine-induced inhibition of adenylate cyclase and cAMP accumulation using specific agonists and antagonists are fully consistent with the conclusion that dopaminergic actions in GH_4ZR_7 cells are mediated by a receptor indistinguishable from the dopamine-D_2 receptor.

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On page 64, line 36:

The discrepancy between the measured affinity of dopamine (FIG. 13C), and the potency of dopamine to inhibit cAMP accumulation ([Figure 13] Figures 13A through 13C) raises the possibility that GH_4ZR_7 cells have "spare" receptors, i.e., a sufficient excess of binding sites to shift the EC_{50} for biological action to values lower than the K_d value. An alternative explanation is that the receptor in membrane preparations has a lower affinity for agonists (but unchanged affinity for antagonists since measured IC_{50} values correlated with K_i values) than in intact cells. Since cytosolic or membrane-associated components present in intact cells are not entirely replaced in membrane binding and adenylate cyclase assay conditions, it is possible that components which allow for optimal function of the dopamine receptor in membrane preparations are lacking. This assertion is supported by the EC_{50} value for inhibition of particulate adenylate cyclase by dopamine (360 nM), which is close to K_i values obtained for dopamine from binding competition experiments (500 nM), but 100-fold higher EC_{50} values (6-8 nM) obtained for inhibition of cAMP levels by dopamine in intact cells. This difference in conditions may explain the observed differences between assays in intact versus particulate preparations. However, affinities of antagonists correlated well with estimated K_i values obtained from cAMP accumulation experiments (FIG. 16), indicating that antagonist binding is similar in membranes and whole cells.

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On page 72, lines 1-12:

[FOOTNOTES

[1]The abbreviations used are: PRL, prolactin; GH, growth hormone; $[Ca^{++}]_i$, cytosolic free calcium concentration; VIP, vasoactive intestinal peptide; TRH, thyrotropin-releasing hormone;

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IBMX, 3-isobutyl-1-methyl xanthine; K_D, equilibrium dissociation constant; EC_{50} (IC_{50}), concentration required to elicit a half-maximal effect (inhibition).

B43 [2]These results have been presented in part at the 71st Annual Endocrine Meetings, Seattle, Wash., Abstract #1278.

[3]Albert, P. R., et al., manuscript in preparation.]

On page 73, line 2:

[Abstract]

A clone encoding a human dopamine-D_2 receptor was isolated from a pituitary cDNA library and sequenced. The deduced protein sequence is 96% identical with that of the cloned rat receptor [Bunzow et al. (1988) Nature 336, 783-787] with one major difference: The human receptor contains an additional 29 amino acids in its putative third cytoplasmic loop. Southern blotting demonstrated B44 the presence of only one human dopamine-D_2 receptor gene. Two overlapping phage containing the gene were isolated and characterized. DNA sequence analysis of these clones showed that the coding sequence is interrupted by six introns and that the additional amino acids present in the human pituitary receptor are encoded by a single exon of 87-basepairs. The involvement of this sequence in alternative splicing and its biological significance are discussed.

On page 76, lines 25-26:

Using the rat brain D_2 receptor cDNA as probe, three partial cDNAs were isolated from a human pituitary library and sequenced. Two oligonucleotide probes based on these sequences were used to isolate a fourth cDNA, $hPitD_2$, which encoded a full-length receptor protein ([Fig. 18] Figures 18A through 18J). The human pituitary receptor contains seven putative transmembrane domains and lacks a signal sequence. Overall, the human and rat nucleotide sequences are 90% B45 similar and show 96% identity at the amino acid level. Several consensus sequences for –linked glycosylation, protein kinase A phosphorylation and palmitoylation (18) are conserved between the human and rat receptors. There are also 18 amino acid differences (including one deletion) between these proteins, and, strikingly, the human pituitary receptor contains an additional 29 amino acids in its putative third cytoplasmic loop.

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On page 78, line 10:

In order to isolate a genomic clone that encoded the –terminus of the human receptor protein, a 118-bp restriction fragment from the cloned rat dopamine-D_2 receptor cDNA (corresponding to amino acid residues 1-39) was used to screen a second genomic library. λHD2G2 was isolated and found to overlap with λHD2G1 by 400 nucleotides ([Fig. 21a] Fig. 21, line a). Together, these phage span 34-kb of the human dopamine-D_2 receptor gene locus, DRD2 (19), and contain the sequence found in the hPitD_2 cDNA plus sequences that extend 15-kb downstream of the polyadenylation signal and 3.7-kb upstream of the translation initiation site. To characterize the intron/exon structure of the gene, a genomic sequencing approach employing oligonucleotide probes and chemical cleavage was used ([Fig. 21b] Fig. 21, line b). Since the divergence of nucleotide sequences between human and rat members of this receptor family is approximately 10% (unpublished observations), we were able to initiate the genomic sequencing relying on hybridization probes and restriction sites that are present in the cloned rat dopamine-D_2 receptor cDNA. Our results demonstrate that the coding portion of the human dopamine-D_2 receptor gene is divided into seven exons ([Fig. 21b] Fig. 21, line b). Interestingly, we found that exon five is 87-bp long and encodes the entire 29 amino acid sequence present in the cloned human pituitary receptor ([Figs. 18] Figures 18A through 18J and Figure 21, line c [21c]). Analysis of the six introns revealed that each contains acceptor and donor sequences that conform to the GT/AG rule (20), as summarized in FIG. 23. The approximate sizes of the introns were based on the results of Southern blotting experiments (data not shown). When compared, the genomic and cDNA sequences were found to differ by only two silent transitions, one at 939 (T to C in the gene) and the other at 957 (C to T in the gene).

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